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Compound mutations in human anion exchanger 1 are associated with complete distal renal tubular acidosis and hereditary spherocytosis

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Missense, nonsense, and frameshift mutations in the human anion exchanger 1 have been associated with inherited distal renal tubular acidosis and hereditary spherocytosis. These two disorders, however, are almost always mutually exclusive. We have found an important and unusual exception: a novel combination of heterozygous E522K and G701D mutations in the anion exchanger 1 manifested as complete distal renal tubular acidosis and severe hereditary spherocytosis in an affected patient. Analysis of protein trafficking and subcellular localization of the wild-type kidney isoform of human anion exchanger 1 and these mutants transfected into MDCK cells showed they formed homodimers or heterodimers with each other. Homodimers of the wild-type and E522K mutant were found at the plasma membrane, whereas the G701D mutant largely remained in the cytoplasm. Heterodimers of either E522K or G701D and the wild-type exchanger were located in the plasma membrane, whereas E522K/G701D heterodimers remained in the cytoplasm. Our study shows that the compound E522K/G701D mutation of human anion exchanger 1 causes a trafficking defect in kidney cells, and this may explain the complete distal renal tubular acidosis of the patient.

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Erythroid anion exchanger 1 (eAE1), also known as band 3, is one of the most abundant proteins in the red blood cell membrane.[1–4] The *AE1* gene is a member of the bicarbonate-anion transporters of the *SLC4* gene family.[5] The central transmembrane domain performs anion exchange in erythrocyte and kidney cells. The erythroid isoform of AE1 (eAE1) is expressed in the red blood cells. The truncated kidney isoform of AE1 (kAE1) is missing the N-terminal 65 amino acids and, beginning at Met-66, is expressed in renal tubular α-intercalated cells.

Missense, nonsense, and frameshift mutations are liable to occur anywhere in the coding sequences of the *AE1* gene in hereditary spherocytosis (HS) patients.[1–4] However, *AE1* mutations, which affect the central transmembrane domain and the short C-terminal tail, are also associated with inherited distal renal tubular acidosis (dRTA), clinically classified as 'complete and incomplete forms', based on the presence or absence of spontaneous metabolic acidosis.[6–8] The AE1 G701D mutation is responsible for Southeast Asian recessive dRTA.[9–15] Combined anemia (not HS) and dRTA in the setting of several instances of compound heterozygosity for AE1 mutations have been reported.[12] Very few patients with HS attributable to AE1 (band 3) mutations are associated with dRTA, and the same applies vice versa,[1–3,8–25] so that HS and dRTA are almost always mutually exclusive.[2,8,25] To the best of our knowledge, the first human exception is a patient in whom AE1 was totally absent, and who presented with complete dRTA and severe HS because of a band 3 Coimbra (V488M) in the homozygous state.[26] An acid loading test revealed that HS patients with band 3 Pribram (C-terminally truncated AE1 protein) or Courcouronnes (S667F) have incomplete dRTA.[27,28] Knockout animal models were developed and reported on in 1996.[29–31] Knockout cattle and mice in which AE1 was totally absent had marked spherocytosis and metabolic acidosis.[29,30,32]

Here, the second human exception (not incomplete dRTA), because of a novel heterozygous E522K mutation (band 3 Kaohsiung) in combination with a heterozygous G701D mutation, exhibited complete dRTA and severe HS.

We investigated the molecular mechanism that causes complete dRTA because of these mutations.

RESULTS

Clinical features

In September 2003, a 7-month-old boy was admitted because he looked extremely pale. His red blood cell indices were as follows: hemoglobin, 4.6 g per 100 ml; mean corpuscular volume, 94 fl; and mean corpuscular hemoglobin concentration, 30.5 g per 100 ml (Table 1). A peripheral blood smear showed marked anisocytosis and spherocytosis, which are typically represented by small, dark-stained red cells without a central pallor (Figure 1a). The reticulocytes accounted for 8.4% of the total red blood cells on the smear (Table 1). An osmotic fragility test yielded an abnormal finding. Arterial blood gas analysis revealed pH 7.02, plasma $HCO3^-$ 4.9 mEq/l, and base excess −23.1 mEq/l. Serum biochemistry showed total bilirubin at 3.7 mg per 100 ml, Na^+ at 138 mmol/l, K^+ at 3.2 mmol/l, and Cl^- at 125 mmol/l. After the child had been given sodium bicarbonate supplements for 1 day, we used an arterial blood gas test (pH 7.22, $HCO3^-$ 9.4 mEq/l), which confirmed complete dRTA with inappropriately high urine pH (UpH 7.78) using the diagnostic criteria of urinary pH over 6.0 despite systemic acidosis. Urine anion gap was + 15 mmol/l. Fractional excretion of bicarbonate and urine to blood (U-B) pCO_2 showed normal findings. This patient was diagnosed with complete dRTA and severe HS, which responded reasonably well to the treatment with daily sodium bicarbonate supplements (6.5 mEq/kg daily) and regular blood transfusions. He had severe splenomegaly, but no nephrocalcinosis or bone disease.

His parents and brother had no anemia, splenomegaly, or renal or bone disease. Both parents and the brother were of normal stature and had normal plasma bicarbonate values (Table 1). The patient's brother and mother had normal bicarbonate reabsorption and renal acidification function according to their fractional excretion of bicarbonate, urine to blood (U-B) pCO_2, urinary anion gap, and ammonium chloride loading tests. His mother and brother had few spherocytes on peripheral blood smears (Figure 1b). Both his brother and mother had very mild HS, according to the guidelines.[4]

Profiling red blood cell membrane protein

To investigate whether the membrane protein defect is associated with the phenotype of HS, we analyzed red cell membrane protein profiles with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The patient's eAE1 protein level was only 23% of that in normal controls (N1–N4) (Figure 1c). This result indicated that a low level of eAE1 protein may be associated with the phenotype of HS and dRTA, which is supported by missense, nonsense, and frameshift mutations of *AE1* tightly associated with HS and dRTA.[1–4,8–25]

Detecting AE1 mutations in genomic DNA

To investigate whether mutations of the *AE1* gene were responsible for the clinical manifestations, we analyzed genomic DNA sequencing. Our results revealed four point mutations in the patient (92 T>C, 166 A>G, 1564 G>A, 2102 G>A, numbered from the *eAE1* mRNA translation

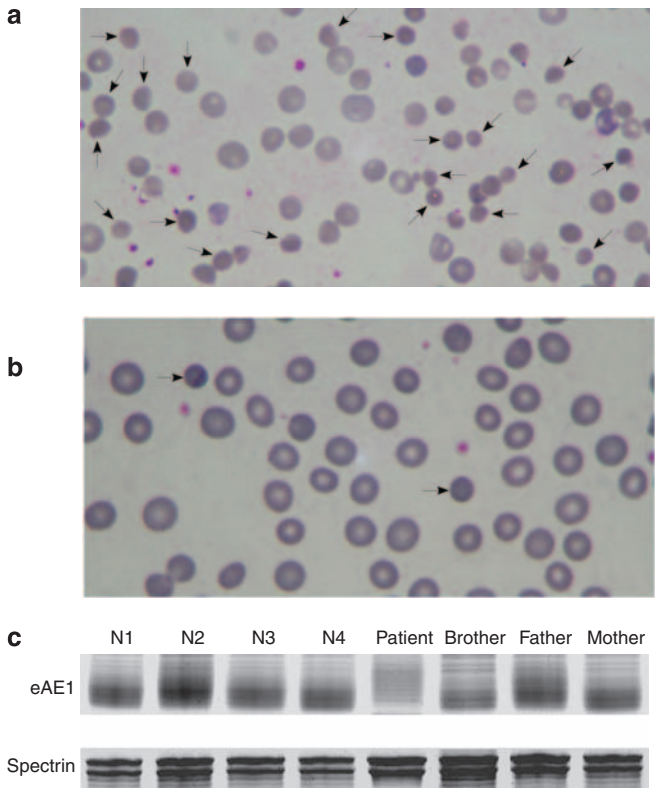


Figure 1 | Red blood cell morphology and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of red blood cell membrane proteins. (a) Peripheral blood smear of the patient shows spherocytosis (arrows) and anisocytosis. **(b)** Peripheral blood smear of the mother shows two spherocytes (arrows). **(c)** SDS-PAGE-separated, Coomassie blue-stained red blood cell ghost polypeptides prepared from intact red blood cells. N1–N4, four normal controls. Spectrin is the internal control to standardize the loading amount.

Table 1 | Laboratory studies and genotypes

	Hb, grams per 100 ml	MCHC, grams per 100 ml	Bil, miligrams per 100 ml	Reticulocyte	Spherocytes	Acidosis	Genotype
Patient	4.6	30.5	3.7	8.4%	Many	+	E522K/G701D
Brother	11.5	35	2	2%	Few	–	E522K/WT
Father	15.7	33.7	0.8	1%	Nil	–	G701D/WT
Mother	12.1	35.6	2.6	1.8%	Few	–	E522K/WT

Bil, bilirubin; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Normal blood pH: 7.35–7.45.

start codon) changing a methionine (ATG) in codon 31 to threonine (ACG), a lysine (AAG) in codon 56 to glutamic acid (GAG), a glutamic acid (GAG) in codon 522 to lysine (AAG), and a glycine (GGC) in codon 701 to aspartic acid (GAC) (Figure 2a and b). The mutations M31T, K56E, and G701D were found in the patient's asymptomatic father, which suggested that this is whence these mutations came, whereas the E522K (band 3 Kaohsiung) mutant was found in his mother and brother, which indicated this mutation had a maternal origin (Figure 2b). Figure 3a illustrates the four mutations of the patient into the proposed topographic models of human eAE1 polypeptide,[3,8,9] and the E522K mutation located near the intracellular boundary of the transmembrane segment TM5. Human kidney AE1 (kAE1), which begins at Met 66,[2,8] lacks codons 31 and 56.

Glutamic acid 522 in AE1 protein is highly conserved in nine mammalian species

Comparing the primary structures of the SLC4A family from nine species showed that glutamic acid 522 and glycine 701 were highly conserved across all nine species (Figure 3b).

Constructing the DNA of kAE1 and its mutants

PCR, as described in Methods, was used to construct human kAE1 and its mutants. Madin-Darby canine kidney (MDCK) cells, which do not express endogenous AE1 and have been used in AE1 trafficking studies,[33–35] were transiently transfected with plasmids to express HA-tagged kAE1 WT, kAE1 E522K, and kAE1 G701D proteins in this study (Figure 4a).

G701D mutants were retained in cytoplasm, but WT and E522K mutants reached the plasma membrane in MDCK cells

Immunofluorescence experiments were done to investigate the subcellular localization of kAE1 E522K and kAE1G701D mutants. Confocal images of transfected MDCK cells showed that HA-tagged kAE1 WT and kAE1 E522K reached the plasma membrane. The membrane localization of kAE1 was confirmed using co-localization with E-cadherin, a basolateral membrane marker (Figure 4b).[35] Moreover, the partial co-localization of the kAE1 WT (or kAE1 E522K) and DsRed-ER, an endoplasmic reticulum (ER) marker,[36] indicated that a certain portion of kAE1 was localized in ER (Figure 4c). However, HA-tagged kAE1 G701D showed no co-localization with E-cadherin in transfected MDCK cells, which suggested that the mutant protein was not present in the plasma membrane (Figure 4b). The kAE1 G701D was partially localized to ER (Figure 4c), which is supported by a report that kAE1 G701D co-localized with the ER marker calnexin and Golgi marker giantin in MDCK cells.[35]

To determine the presence or absence of wild-type basolateral targeting as a property of kAE1 E522K, transiently transfected MDCK cells were examined on filter supports as polarized cells. Similar to kAE1 WT, kAE1 E522K was found at the basolateral membrane after cellular polarization (Figure 4d). By contrast, kAE1 G701D was retained intracellularly in polarized cells. Taken together, the results

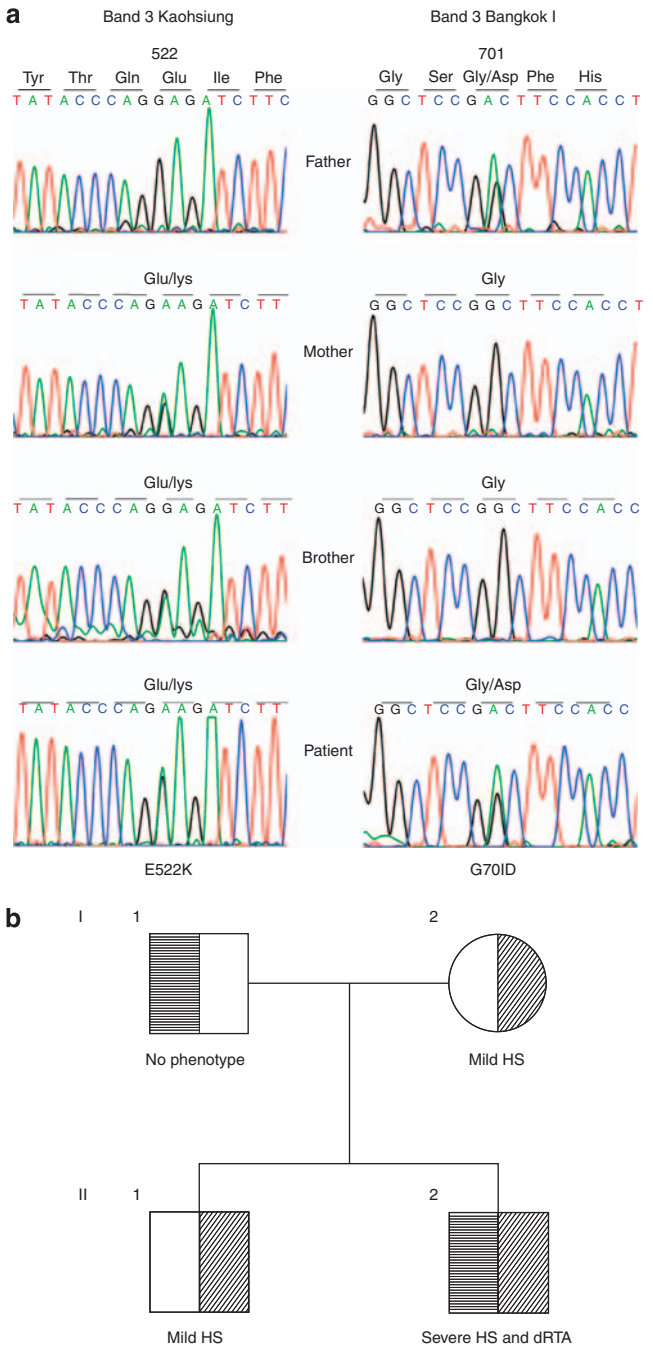
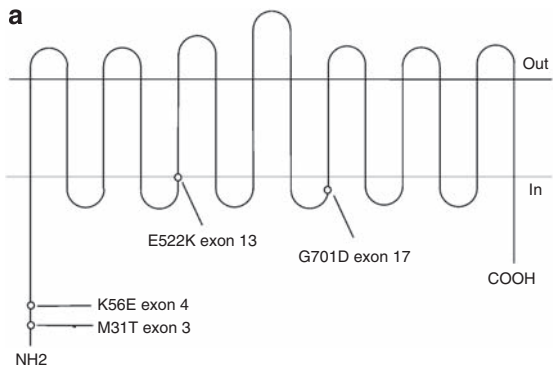


Figure 2 | DNA sequence profiles and family pedigree. **(a)** Identifying the *AE1* (band 3) mutations. DNA sequence profiles of the regions adjacent to codons 522 and 701 for the father (I:1), the mother (I:2), the brother (II:1), and the patient (II:2). In the patient and his mother and brother, the band 3 Kaohsiung (E522K) substitutes glutamic acid (GAG) in codon 522 for lysine (AAG). In the patient and his father, the band 3 Bangkok I (G701D) changes glycine (GGC) in codon 701 to aspartic acid (GAC). **(b)** Family pedigree. Allele band 3 Kaohsiung is indicated by cross hatching, allele band 3 Bangkok I by horizontal symbols.

confirmed that G701D mutants were retained in the cytoplasm, whereas the WT and E522K mutants reached the basolateral membrane in MDCK cells. Two possible mechanisms that might have caused AE1 mutants to be



b

	SLC4A	522	
P02730IB3AT_Human	1	SRYTQEIFSFLISLIFIYETFSKLIKIFQDHPLQK	551
088343IS4A4_Mouse	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
Q9JI66IS4A4_Rat	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
Q9JY6R1IS4A4_Human	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
Q9GL77IS4A4_Bovin	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
Q4U116IS4A4_Pig	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
Q9XSZ4IS4A4_Rabit	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	615
01314IS4A4_Ambti	4	TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINS	570
Q32MA710Q32MA7_Human	5	TRFTEEGFSTLISFIFIYDAIKKMIGAFKYYPINM	603
Q6U841IS4A10_Human	10	TRFTEEAFASLICIIFIYEALEKLFELSEAYPINM	655
Q32LP4IS4A10_Bovin	10	TRFTEEAFASLICIIFIYEALEKLFELSEAYPINM	654
Q5DTL9IS4A10_Mouse	10	TRFTEEAFASLICIIFIYEALEKLFELSETYPINM	655
Q80ZA5IS4A10_Rat	10	TRFTEEAFASLICIIFIYEALEKLFELSEAYPINM	654
Q2Y0W81Q2Y0W8_Human	8	TRFTEEAFASLICIIFIYEAIEKLIHLAETYPIHM	624
Q9Y6M7IS4A7_Human	7	TRFTEEAFAALICIIFIYEALEKLFDLGETYAFNM	754
Q96Q91IB3A4_Human	9	TRFTEEGFCALISLIFIYDAVGKMLNLTHTYPIQK	556
Q9GKY1IB3A4_Rabit	9	TRFTEEGFCALISLIFIYDAVGKMLNLAHAYPIQR	530
Q8K4V2IB3A4_Rat	9	TRFTEEGFCALISLIFIYDAVGKMLNLIRAYPIQR	528
P04919IB3AT_Mouse	1	SRYTQEIFSFLISLIFIYETFSKLIKIFQDYPLQQ	570
P23562IB3A2_Rat	1	SRYTQEIFSFLISLIFIYETFSKLIKIFQDYPLQE	568
P15575IB3A2_Chick	1	SRYTQEIFSFLISLIFIYETFAKLVTIFEAHPLQQ	564
P04920IB3A2_Human	2	SRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHG	854
P48746IB3A2_Rabit	2	SRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHG	850
P13808IB3A2_Mouse	2	SRFTQEIFAFLISLIFIYETFYKLVKIFQEHPLHG	850
P23347IB3A2_Rat	2	SRFTQEIFAFLISLIFIYETFYKLVKIFQEGPLHG	851
Q9Z0S8IB3A2_Cavpo	2	SRFTQEIFAFLISLIFIYETFYKLVKIFQEGPLHG	851
P16283IB3A3_Mouse	3	SPFTQEIFAFLISLIFIYETFHKLYKVFTEHPLLP	853
P23348IB3A3_Rat	3	SPFTQEIFAFLISLIFIYETFHKLYKVFTEHPLLP	853
P48751IB3A3_Human	3	SPFTQEQFAFLISLIFIYETFYKLYKVFTEHPLLP	854
018917IB3A3_Rabit	3	SPFTQEIFAFLISLIFIYETFHKLYKVFTEGPLLP	855
P32847IB3AT_Oncmy	1	SRFTQEIFSILISLIFIYETFSKLGKIFKAHPLVL	540

Figure 3 | Schematic model and sequence comparisons. (a) Schematic model of eAE1. Codon substitutions encoded by the four mutations detected in the indicated exons of the AE1 gene are mapped into a topographic representation of human eAE1 polypeptide modified by Tanphaichitr *et al.*[9] The Band 3 Kaohsiung mutation (AE1 E522K) causes a substitution of a glutamic acid in the intracellular boundary of the transmembrane segment TM5. The two transmembrane domains with E522K and G701D mutations are within the region of less certain topography. **(b)** Sequence comparisons for eAE1 (band 3, SLC4A1, P02730) and related anion exchanger proteins. The codon AE1 522 glutamic acid was conserved in the 31 related proteins (rectangle), which were retrieved from the UniProtKB/Swiss-Prot database (http://www.expasy.org) and aligned using the program ClustalW (http://www.ebi.ac.uk/clustalw/). The primary accession numbers and entry names used in the UniProtKB/Swiss-Prot database are in the first column. The SLC4 family numbers are in the second column, and the amino acid sequences of the 31 proteins are in the third column. *Conserved amino acids.

retained in the cytoplasm are AE1 protein misfolding that prevented their dimerization or their moving to the cell surface, which increases the degradation and consequently the functional loss of AE1.[8,9,15,33–35]

The WT formed heterodimers with the E522K and G701D mutants, which helped them reach the cell surface

To clarify which mechanism caused these two mutants to be retained in the cytoplasm, co-immunoprecipitation was used to examine the co-expression of HA-tagged and flag-tagged AE1 constructs in the interactions between kAE1 WT, kAE1 E522K, and kAE1 G701D. Cell lysates were first immunoprecipitated with anti-HA antibody and the precipitates were analyzed using immunoblotting with anti-flag antibody. Heterodimers formed between flag-kAE1 WT and HA-kAE1 WT, flag-kAE1 WT and HA-kAE1 E522K, and flag-kAE1 WT and HA-kAE1 G701D (Figure 5). This was supported by the co-localization of flag-kAE1 WT and HA-kAE1 mutants (Figure 6a). On the basis of the quantitative analysis described in Methods and Figure 6b, co-expression of flag-kAE1 WT and HA-kAE1 WT showed that the cell peripheral expression for both tagged proteins was about $42.2 \pm 14.7\%$ for HA-tagged WT and $46.8 \pm 14.8\%$ for flag-tagged WT ($n = 10$) (Figure 6c). Similarly, co-expression of flag-kAE1 WT and HA-kAE1 E522K or HA-kAE1 G701D revealed obvious cell peripheral co-expression and co-localization of kAE1 proteins (Figure 6a). The cell peripheral expression showed no significant difference ($P > 0.05$) between co-expression of WT/WT and E522K/WT or G701D/WT (Figure 6c).

Intact KAE1 WT/WT cells were biotinylated with NHS-SS-biotin to investigate the cell surface expression of kAE1 in

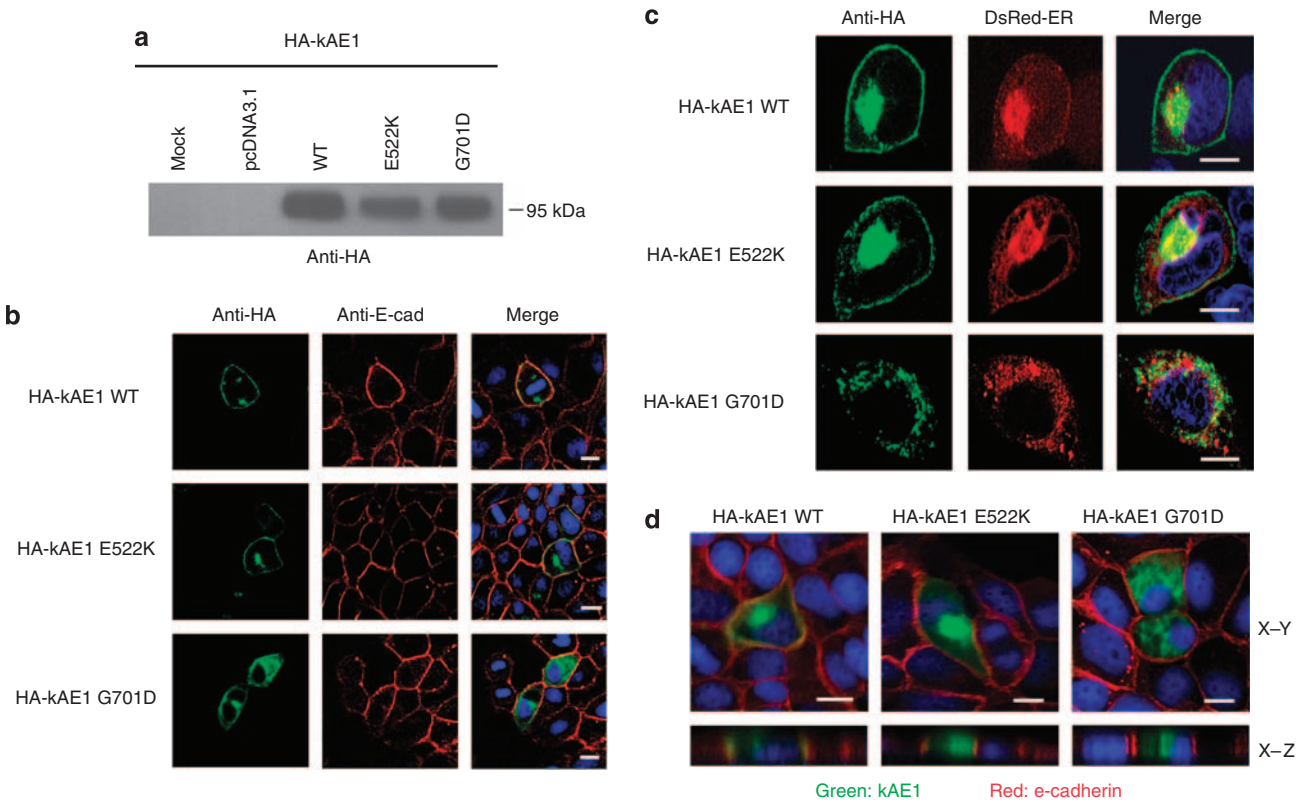


Figure 4 | Expression and cellular localization of wild-type and mutant kAE1 fusion proteins in MDCK cells. **(a)** Cells transiently transfected with mock, pcDNA3.1 vector, HA-kAE1 WT, HA-kAE1 E522K, and HA-kAE1 G701D. The cell lysates were immunoblotted with anti-HA antibody. **(b)** Cellular localization of kAE1, kAE1 E522K, kAE1 G701D, and E-cadherin. Transfected MDCK cells expressing either HA-tagged kAE1 WT, E522K, or G701D were grown on glass coverslips, fixed, permeabilized, and incubated with mouse anti-HA and rabbit anti-E-cadherin antibody. After the cells were washed, they were incubated with goat anti-mouse FITC and goat anti-rabbit rhodamine, used to visualize kAE1 and E-cadherin, respectively. After they had been stained with DAPI, the localization of fluorescently labeled proteins was visualized, using a confocal microscope. E-cadherin is a basolateral membrane marker. **(c)** Cellular localization of kAE1, kAE1 E522K, kAE1 G701D, and DsRed/ER. Transfected MDCK cells expressing DsRed/ER and either HA-tagged kAE1 WT, E522K, or G701D were grown on glass coverslips. The cells were then fixed, permeabilized, and incubated with mouse anti-HA antibody. After they were washed, the cells were stained with DAPI and goat anti-mouse antibody bound to FITC. The kAE1 proteins were then observed, using a confocal microscope. The endoplasmic reticulum was visualized with DsRed-ER localization vector. **(d)** Cellular localization of kAE1 and mutants in polarized MDCK cells. Transiently transfected MDCK cells expressing kAE1 WT, E522K, or G701D, grown on polycarbonate filters, were detected using anti-HA antibody (green), whereas basolateral membrane was marked with E-cadherin (red) and nucleus was stained with DAPI (blue). Yellow staining corresponds to overlap between green and red labeling. X–Y shows middle section of the cells. X–Z corresponds to side view of the cells. Anti-E-cad, anti-E-cadherin antibody; Anti-HA, anti-HA antibody. Bar represents 10 μm. Results shown are typical of three independent experiments.

co-transfected MDCK cells (Figure 7, lane B). This indicated the presence of kAE1 at the cell surface, which was determined to be $41.7 \pm 5.3\%$ ($n = 3 \pm$ s.d.) of the total kAE1 expression. However, the percentage of the total kAE1 protein on the cell surface was $51 \pm 6\%$, determined by cell surface biotinylation in stably transduced non-polarized MDCK cells.[33] The difference in percentages of WT kAE1 at the cell surfaces of transiently and stably transfected MDCK cells might have been because the expression level is usually very high in a transient transfection assay, which overloads the folding and assembling capacity of ER and Golgi complex.[37]

Similarly, co-expression of WT and E522K or G701D revealed obvious cell surface expression of kAE1 proteins (Figure 7). The cell surface expression was not significantly different ($P > 0.05$) from the co-expression of WT/WT and E522K/WT or G701D/WT. These results indicated that kAE1 WT prevented the intracellular retention of kAE1 G701D, which allowed the mutant protein to move from the ER to the plasma membrane through hetero-oligomerization. The above results not only defined the recessive characteristic of these two mutations, but also explained the normal phenotype of the patient's father and mother.

E522K formed a homodimer and moved to the cell surface

Co-immunoprecipitation was also used to investigate the formation of the E522K homodimer. Flag-tagged kAE1 E522K was detected in an anti-HA antibody immunoprecipitated complex that co-expressed HA-tagged kAE1 E522K and flag-tagged kAE1 E522K (Figure 5, lane 5), which showed that a homodimer of E522K had been formed. Furthermore, co-expression of flag-kAE1 E522K with HA-kAE1 E522K in MDCK cells showed peripheral expression of $31.1 \pm 9.7\%$ ($n = 10$) (Figure 6a and c, anti-HA). These results were

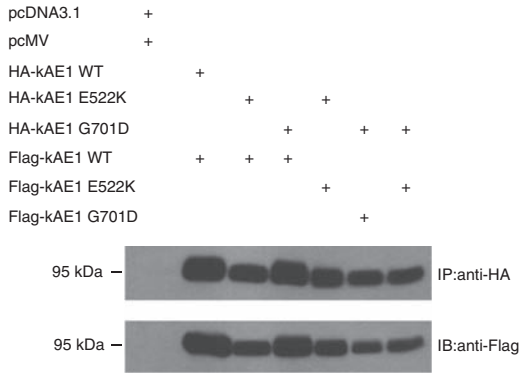


Figure 5 | Co-immunoprecipitation and immunoblotting. MDCK cells co-transfected with differentially tagged proteins (HA- or flag-) were lysed. The cell lysates were incubated with mouse anti-HA monoclonal antibody and agarose ligand. The bound proteins were eluted with denatured buffer, and HA-tagged proteins were detected using immunoblotting with mouse anti-HA monoclonal antibody (IP) or rabbit anti-flag polyclonal antibody (IB). After the membranes were washed, they were incubated with HRP-conjugated second antibodies. IP, immunoprecipitation; IB, immunoblotting. Results shown are typical of three independent experiments.

significantly ($^*P = 0.032$) lower compared with peripheral expression ($42.2 \pm 14.7\%$) ($n = 10$) of WT homodimer (Figure 6c). Similarly, peripheral expression of E522K was mildly lower (Figure 4c). In the biotinylation experiment, E522K homodimer cell-surface expression ($20.3 \pm 6.2\%$) ($n = 3$) was significantly lower ($^*P = 0.011$) compared with WT homodimer expression ($41.7 \pm 5.3\%$) ($n = 3$) (Figure 7).

E522K mutant formed heterodimer with G701D mutant but did not help it reach the cell surface

Lysates from MDCK cells co-expressing either HA-kAE1 G701D/flag-kAE1 G701D or HA-kAE1 G701D/flag-kAE1 E522K were immunoprecipitated using anti-HA antibody, and then immunoblotted using anti-flag antibody. kAE1 G701D/G701D and kAE1 E522K/G701D heterodimers were formed (Figure 5, lanes 6–7). Co-expressed HA-kAE1 G701D with either flag-kAE1 G701D or flag-kAE1 E522K revealed obvious intracellular retention and co-localization of both mutant kAE1 proteins (Figure 6a), with average percentages of relative cell peripheral expression of 0 and 0% ($n = 10$), respectively (Figure 6c, anti-HA). Cell peripheral localization of these mutant proteins was significantly ($P < 0.0001$) lower compared with co-expressed flag-kAE1 WT with HA-kAE1 WT. Taken together, all these results suggested that 100% of the kAE1 G701D/G701D and E522K/G701D mutant proteins were retained in the cytoplasm. Moreover, in the cell-surface biotinylation experiment, the kAE1 G701D mutant was not detected in the biotinylated fraction in MDCK cells co-transfected with kAE1 G701D/G710D and G701D/E522K ($0.5 \pm 0.4\%$ and $0.3 \pm 0.3\%$, respectively, $n = 3$) (Figure 7, lane B). The results indicated that kAE1 E522K and kAE1 G701D did not help each other move to the cell surface, which resulted in their intracellular retention; this helps explain the dRTA phenotype of the patient. Similarly, the intracellular retention of kAE1 G701D/G701D mutant protein was consistent with other trafficking studies.[9,15,35]

The kAE1 E522K and G701D mutations destabilized themselves

To clarify whether retaining kAE1 mutants in the cytoplasm destabilizes themselves, the protein expression levels and stability were monitored at 0, 4, 7, and 11 h after treating cells with cycloheximide, a protein biogenesis inhibitor (Figure 8a). At 0 h, the level of kAE1 mutant proteins were lower than that of kAE1 WT (Figure 8a), and the expression levels of kAE1 WT and kAE1 mutants (kAE1/actin ratios) were set at 100% (Figure 8b). The immunoblotting showed that more rapid reduction in kAE1 E522K and G701D expression than in kAE1 WT was most likely caused by a more rapid turnover of kAE1 mutants (Figure 8a and b). In these experiments, the half-life of both kAE1 WT and kAE1 E522K was about 11 h, whereas the half-life of kAE1 G701D was about 5 h (Figure 8b). These results were similar to reports that kAE1 S773P and kAE1 SAO have a half-life of about 5 h in human embryonic kidney cells (HEK 293 cells),[15,38] and that kAE1 G701D has a half-life of about 6 h in MDCK cells.[35] Misfolded mutant proteins did not reach the cell surface, and then the cellular quality control system degraded the mutants.[39,40] ER- and lysosome-associated degradation may explain the 5-h half-life of the G701D mutant and its retention in ER (Figure 4c) and co-localization with lysosomes (results not shown); this agrees with a report that proteosome- and lysosome-dependent degraded E258K aquaporin-2 caused congenital nephrogenic diabetes insipidus.[41]

DISCUSSION

The patient in this study is the second human being with complete dRTA and severe HS because of a novel heterozygous E522K mutation (band 3 Kaohsiung) combined with a heterozygous G701D mutation. The band 3 Kaohsiung mutation may cause the substitution of a glutamic acid in the intracellular boundary of the fifth transmembrane segment (TM5) (Figure 3a). The substitution replaces glutamic acid, a negatively charged hydrophilic amino acid, with lysine, a positively charged hydrophilic residue. In addition to hydrophobic segments, the distribution of positively charged amino acids seems to be an important determinant in eukaryotic membrane topology.[42]

The recessive dRTA mutants, such as homodimer S773P and ΔV850, are impaired when they shift to the plasma membrane and localize to the ER in non-polarized and polarized MDCK cells, respectively. Similarly, the other recessive mutant, kAE1 G701D, localized to the ER and Golgi marker in both non-polarized and polarized cells.[35,43] However, this study revealed that the novel homodimer kAE1 E522K also moved to the plasma membrane (Figures 4b, d, 6a and 7). The effect of the novel missense recessive mutation E522K on the biosynthesis of kAE1 was characterized in

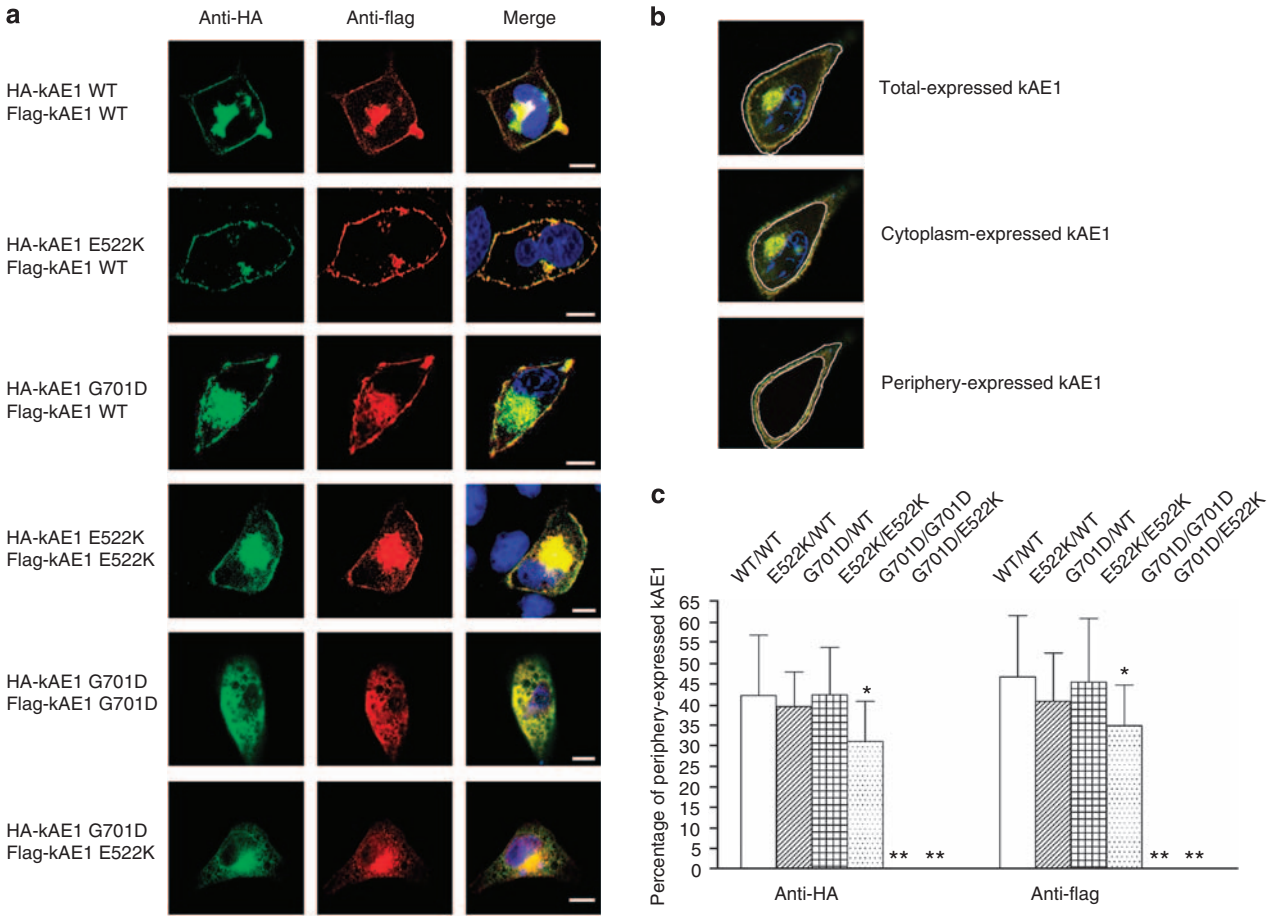


Figure 6 | Co-localization of kAE1 E522K, kAE1 G701D, and wild-type AE1 at the plasma membrane. (a) Non-polarized MDCK cells transiently co-transfected with six combinations: kAE1 WT/WT, E522K/WT, G701D/WT, E522K/E522K, G701D/G701D, and G701D/E522K. At 1 day after co-transfection, the cells were fixed with 3.7% paraformaldehyde, permeabilized using incubation in 0.4% Triton X-100, blocked with 2% goat serum, and then stained with mouse anti-HA monoclonal and rabbit anti-flag polyclonal antibodies that recognize HA-kAE1 and flag-kAE1, respectively. After the cells were washed, they were incubated with goat anti-mouse FITC and goat anti-rabbit rhodamine to visualize kAE1 and E-cadherin, respectively. After the cells were stained with DAPI, the localization of fluorescently labeled proteins was visualized using a confocal microscope. Anti-HA, anti-HA antibody; Anti-flag, anti-flag antibody. Bar represents 10 μm. Results shown are typical of three independent experiments. **(b)** Quantitative procedure. Top panel: the intensity of kAE1 immunofluorescence of the area of the entire cell (area I) was measured in the AE1-transfected MDCK cells. Middle panel: the intensity of kAE1 immunofluorescence of the area within the bounds of the line just inside the membrane (area II) was measured. Lower panel: the difference between the values from area I and area II represents the peripheral kAE1. **(c)** Quantitative analysis for immunofluorescence imaging. The bar graph shows the percentage of peripheral co-expression of kAE1 WT/WT, E522K/WT, G701D/WT, E522K/E522K, G701D/G701D, and G701D/E522K from MDCK cells. The immunofluorescence image was analyzed using NIH image software. Error bars represent s.d. ($n = 10$). *, ** significant decreases compared with WT/WT ($P < 0.05$ and $P < 0.01$, respectively; two-tailed unpaired Student's *t*-tests).

experiments of co-transfected MDCK cells; they revealed that the kAE1 E522K mutants formed homo-oligomers and less efficiently moved to the plasma surface (Figures 5, 6c and 7).

The kAE1 E522K/WT heterodimer moved to the plasma membrane, but the kAE1 E522K mutant did not reach the cell surface in the presence of kAE1 G701D (Figures 6a and 7). These would explain the normal phenotype of the mother with the genotype of kAE1 WT/E522K and the dRTA phenotype of the patient with kAE1 E522K/G701D. kAE1 WT protein helped the mutant kAE1 G701D reach the cell surface, but kAE1 E522K did not help it reach the plasma membrane, which is similar to the reports that kAE1 SAO and kAE1 S773P formed heterodimers with kAE1 G701D but did not help it reach the plasma membrane.[15,44] Taking all these findings together, we conclude that kAE1 WT protein forms heterodimers with kAE1 E522K, G701D, SAO, and S773P mutants and helps them reach the cell surface.[15,44]

In conclusion, HS and dRTA are almost always mutually exclusive.[2,8,25] However, our patient was the second known human being with complete dRTA and severe HS, a comorbidity caused, in this case, by the synergistic effects of a symptomless band 3 Bangkok I allele and a novel band 3 Kaohsiung allele that generated only slight HS with a band 3 deficiency.

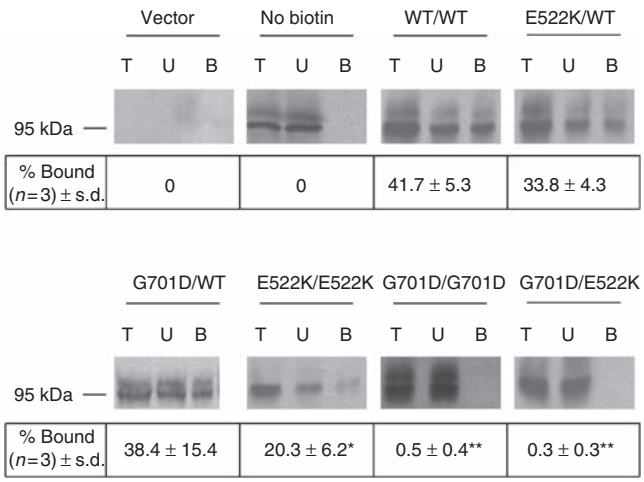


Figure 7 | Surface biotinylation on MDCK cells co-transfected with combinations of kAE1 WT, E522K, and G701D. Transfected MDCK cells were surface-biotinylated by incubating them twice with NHS-SS-biotin reagent in borate buffer. The cells were quenched of any unreacted reagent, and the biotinylated proteins in the cell lysates were captured using streptavidin–agarose beads. Immunoblotting with anti-HA antibody showed the amount of kAE1 in the total (lane T), unbound to streptavidin beads (lane U) and bound to streptavidin beads (lane B). The percentage of the cell surface expression was quantitated by scanning lanes T and B and analyzing the images with NIH image software ($n = 3$). *, ** significant decreases compared with WT/WT *, ** significant decreases compared with WT/WT. ($P < 0.05$ and $P < 0.01$, respectively; two-tailed unpaired Student's *t*-tests).

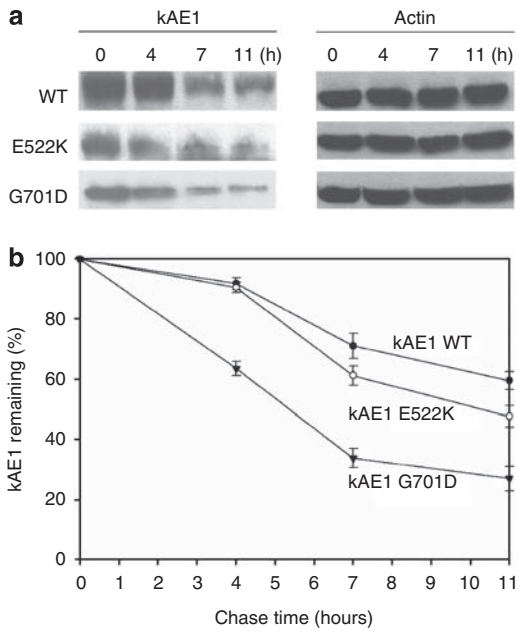


Figure 8 | Protein stability experiments with kAE1 WT, kAE1 E522K, and kAE1 G701D. (a) The transfected MDCK cells expressing HA-kAE1 WT, HA-kAE1 E522K, or HA-kAE1 G701D grew on 12-well tissue culture plates for 24 h. At each time checkpoint, 0, 4, 7, and 11 h after cycloheximide (a protein biogenesis inhibitor) was added, the cell lysates were immunoblotted with anti-HA and anti-actin antibodies (an internal control). **(b)** The results of quantitative densitometric scanning and analysis using NIH software are shown in the graph. Each data point is the mean derived from four measurements at each time point. Error bars represent the s.d. ($n = 4$).

MATERIALS AND METHODS

Erythrocyte membrane protein analysis

The erythrocyte ghosts were prepared using methods previously described.[45] Red blood cell membrane proteins were analyzed using Coomassie blue-stained 10% SDS-PAGE.[46] Image analysis software (NIH Image; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used for densitometric measurement to quantify the protein content of red cell membrane proteins.[46,47]

AE1 genomic DNA analysis

Using Genomic DNA as templates, all exons of the *AE1* gene were amplified using PCR with pairs of intronic primers flanking the exons. The primers were designed based on a previously described method.[18]

Sequence comparisons for AE1 (SLC4A1) and related proteins

Amino acid sequences of all 31 currently known sequences of SLC4A1, SLC4A2, SLC4A3, SLC4A4, SLC4A5, SLC4A7, SLC4A8, SLC4A9, and SLC4A10 from humans and mammals were retrieved from UniProt/TrEMBL (http://www.expasy.org) and aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/).

Constructing WT full-length cDNA and mutants

Full-length cDNA was constructed as previously described.[47]. Total RNA was isolated from the buffy coat of the patient's blood using acidic guanidinium isothiocyanate TRI-reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse-transcribed into cDNA using a kit (ImProm-II Reverse Transcription System; Promega, Madison, WI, USA) according to the manufacturer's instructions. Using cDNA as template, four fragments were amplified by PCR using the four primers (1) kAE1 RsrII-F: 5′-GACACGGTCCGATGGACGAAAGAACCAG-3′ (RsrII site and 196–212, start codon of the kidney isoform); (2) D: 5′-TCCGA-CACTCCCATCTGGTT-3′ (1316–1297); (3) E: 5′-AGACTC-CAGCTTCTACAAGG-3′ (1062–1081); and (4) RsrII-R: 5′-GACACGACCGTCACACGGCATGGCCACT-3′ (RsrII site and 2719–2736). The PCR products were separated, purified, and ligated into the yT&A vectors. The yT&A vectors were sequenced and subcloned into the pcDNA3.1-HA-CPO and pCMV2.0-flag-CPO (modified by adding an RsrII restriction enzyme site) (Invitrogen), thus yielding six plasmid constructs expressing kAE1, kAE1 E522K, and kAE1 G701D fused with either HA or flag tag at its N-terminus.

Cell culture and transient transfection

Madin-Darby canine kidney cells were maintained in DMEM with 10% cow bovine serum, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in 5% CO_2. The plasmids were transiently transfected using a reagent (Lipofectamine; Invitrogen).[47] Polarized MDCK cells were obtained by growing confluent cells on Transwell polycarbonate filters (Corning Headquarters, Corning, NY, USA) for 4–5 days.

Immunoblotting analysis

Proteins were separated using 10% SDS-PAGE and then transferred to a PVDF membrane, which was incubated with rabbit anti-flag antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) or

mouse anti-HA monoclonal antibody (Santa Cruz Biotechnology). After it had been washed, the membrane was incubated with HRP-conjugated second antibody. Immunoreactivity was detected using an ECL chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence analysis

MDCK cells,[48] transfected with DsRed-ER (Clontech, BD Biosciences, Palo Alto, CA, USA) and either plasmids expressing kAE1 WT, E522K, or G701D fused HA epitope, were left to grow on cover slips or Transwell polycarbonate filters, fixed with 3.7% paraformaldehyde in PBS or 100% methanol, permeabilized by incubating them in 0.4% Triton X-100, and blocked with 2% goat serum for 15 min. The cells were then incubated with the mouse anti-HA monoclonal antibody (Santa Cruz Biotechnology), and E-cadherin antibody (Transduction Laboratories, Lexington, KY, USA) at 4 °C overnight. After they had been washed, the cells were incubated with secondary antibody. After the cells had been stained with DAPI, the localization of fluorescently labeled proteins was visualized using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Thornwood, NY, USA).

Co-immunoprecipitation

MDCK cells (co-transfected with plasmids expressing WT/WT, WT/E522K, WT/G701D, E522K/E522K, G701D/G701D, E522K/G701D fused with flag and HA epitope) were homogenized in RIPA lysis buffer.[49,50]. The supernatant was incubated with 10 µl of mouse anti-HA monoclonal antibody and agarose ligand (Millipore, Billerica, MA, USA) and rotated in a cold room overnight. After they had been washed thrice, the proteins were eluted with 1 × denaturizing buffer. Protein lysate (40 µg) was analyzed using SDS-PAGE and then transferred to a PVDF membrane, which was then analyzed using immunoblotting as described above.

Co-localization

MDCK cells (co-transfected with plasmids expressing WT/WT, WT/E522K, WT/G701D, E522K/E522K, G701D/G701D, and E522K/G701D fused with flag and HA epitope) were incubated as described above using the mouse anti-HA antibody and rabbit anti-flag polyclonal antibody. After they had been washed, the cells were incubated with secondary antibody. The localization of fluorescently labeled proteins was examined using confocal microscopy. Quantitative analysis for immunofluorescence imaging was performed as described elsewhere[51] and in the legend of Figure 6b.

Cell surface biotinylation

The protocol of cell surface biotinylation was adapted from the previous studies.[15,33]. Briefly, MDCK cells were co-transfected with plasmids as described above. Parallel control cells were co-transfected with vectors only (pcDNA3.1-HA and pCMV2.0-flag). The cells were treated twice with 1 ml of 0.5 mg/ml EZ-Link NHS-SS-biotin (Pierce Biotechnology, Rockford, IL, USA) in borate buffer for 15 min at 4 °C. The cells were lysed with RIPA buffer. After they had been washed, ImmunoPure (Pierce Biotechnology) bead-bound protein was eluted by adding SDS sample buffer. The content of kAE1 in the total bound and unbound fractions were analyzed using immunoblotting with anti-HA antibody. The scanning images were analyzed using NIH image software as previously described.[47,48]

Protein stability analysis

MDCK cells, transfected with plasmids expressing kAE1 WT, E522K, or G701D fused HA epitope, were incubated on 12-well tissue culture plates for 24 h. At each time check point 0, 4, 7, and 11 h after adding 25 µg/ml of cycloheximide (Calbiochem-Novabiochem International, La Jolla, CA, USA), the cell lysates were separated using SDS-PAGE, analyzed using immunoblotting with anti-HA and anti-actin antibodies (as an internal control to standardize the loading amount), and then analyzed using NIH image software as previously described.[47,48]

DISCLOSURE

All the authors declared no competing interests.

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